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# Identification and Initial Characterization of Three Novel Cyclin-related Proteins of the Human Malaria Parasite *Plasmodium falciparum*\*

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The molecular mechanisms regulating cell proliferation and development during the life cycle of malaria parasites remain to be elucidated. The peculiarities of the cell cycle organization during *Plasmodium falciparum* schizogony suggest that the modalities of cell cycle control in this organism may differ from those in other eukaryotes. Indeed, existing data concerning *Plasmodium* cell cycle regulators such as cyclin-dependent kinases reveal structural and functional properties that are divergent from those of their homologues in other systems. The work presented here lies in the context of the exploitation of the recently available *P. falciparum* genome sequence toward the characterization of putative cell cycle regulators. We describe the *in silico* identification of three open reading frames encoding proteins with maximal homology to various members of the cyclin family and demonstrate that the corresponding polypeptides are expressed in the erythrocytic stages of the infection. We present evidence that these proteins possess cyclin activity by demonstrating either their association with histone H1 kinase activity in parasite

extracts or their ability to activate PpPK5, a *P. falciparum* cyclin-dependent kinase homologue, *in vitro*. Furthermore, we show that RINGO, a protein with no sequence homology to cyclins but that is nevertheless a strong activator of mammalian CDK1/2, is also a strong activator of PpPK5 *in vitro*. This raises the possibility that “cryptic” cell cycle regulators may be found among the 50% of the open reading frames in the *P. falciparum* genome that display no homology to any known proteins.

Four to eight thousand people, most of them children in sub-Saharan Africa, die every day from malaria. The spread of drug resistance in *Plasmodium falciparum*, the parasitic protozoan responsible for the vast majority of lethal malaria cases, is a cause for grave concern with respect to disease control and renders the development of novel chemotherapeutic agents an urgent task (1). The identification of potential targets is now greatly facilitated by the availability of genomic data bases (2) (see www.PlasmoDB.org) resulting from an international effort that led to the recent publication of the entire *P. falciparum* genome sequence (3).

The life cycle of malaria parasites consists of a succession of developmental stages, some of which are characterized by intense cell proliferation, whereas in others the cell cycle is arrested. *P. falciparum* infection of the human host is initiated by injection of cell cycle-arrested sporozoites into the bloodstream by an infected *Anopheles* mosquito. The sporozoites rapidly gain the liver and invade hepatocytes, where asexual multiplication (exoerythrocytic schizogony) occurs, leading to the production of several thousand merozoites. These are released into the bloodstream and invade erythrocytes, which become the site of another round of asexual multiplication, producing 8–24 new merozoites/infected red blood cell. This phase of the infection (erythrocytic schizogony) is responsible for malaria pathogenesis. The molecular mechanisms through which some of the merozoites, upon red blood cell invasion, arrest their cell cycle and differentiate into male or female gametocytes instead of initiating a new round of schizogony are not understood. Ingestion of gametocytes by a mosquito is required for transmission to the insect vector, in the midgut of which these cells mature into gametes; for the male gametocytes, this requires sudden release from cell cycle arrest and three rounds of cell division to produce eight gametes. Fertilization in the mosquito midgut then yields a zygote, the only diploid stage in the life cycle of the parasite. Meiotic reduction

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AJ416696, AJ416697, and AJ416698.

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occurs in the ookinete, the motile form into which the zygote rapidly develops. This ookinete crosses the peritrophic membrane and the midgut epithelium and immobilizes at the epithelium basal lamina. There it undergoes further development into an oocyst, a site of intense asexual multiplication producing several thousand sporozoites, which are cell cycle-arrested and accumulate in the salivary glands of the mosquito, ready to be injected into a vertebrate host during a subsequent blood meal (see [www.malaria.org](http://www.malaria.org) for more information on malaria).

Obviously, the parasite must tightly regulate its cell division status in response to its environment to complete its life cycle. The organization of the *Plasmodium* cell cycle in the different developmental stages and the molecular mechanisms through which its progression is controlled are far from being understood (reviewed in Refs. 4 and 5). Erythrocytic schizogony has received more attention from researchers than other dividing stages of the life cycle of the parasite, because (i) this process is responsible for pathogenesis and (ii) it is comparatively easier to obtain biological material from infected red blood cells cultured *in vitro* than from other stages. A clear correspondence between cellular events occurring during *Plasmodium* schizogony and the G<sub>1</sub>, S, G<sub>2</sub>, and M phases of the "classical" cell cycle has not been established (6). The invading merozoite is presumably in G<sub>1</sub>, and DNA synthesis is initiated in the trophozoite ~18 h post-invasion. Nuclear divisions ensue in the absence of cytodieresis, resulting in the formation of a schizont; nuclei in a given schizont appear to divide asynchronously (7), which suggests that cell cycle progression is not dictated solely by fluctuations in the cytoplasmic abundance of cell cycle regulators. It is not clear whether a strict succession of S phases and nuclear divisions occurs, or whether segregation of newly replicated genomes into individual nuclei takes place in a different way. Plasmodial cell multiplication displays other atypical features, such as the absence of chromosome condensation, the maintenance of the nuclear membrane (cryptomitosis), and the presence of intranuclear spindles.

The asynchrony of nuclear division in a given schizont renders biochemical analysis of cell cycle progression very difficult. A possible approach to gaining insight into molecular processes of cell cycle control in *Plasmodium* is to study the function, subcellular localization, and other properties of putative regulators such as cyclins and cyclin-dependent protein kinases (CDKs).<sup>1</sup> Several enzymes related to CDKs have been characterized to date in *P. falciparum* (reviewed in Ref. 8). Two of these enzymes have been shown to be activated by association with cyclins: PPK5, a putative homologue of CDK1 (9) that can be activated *in vitro* by a variety of cyclin-related proteins including mammalian cyclin H and p25, and Pfcyc-1, the only *Plasmodium* cyclin characterized so far (10). Hence PPK5 appears to display promiscuity in its cyclin requirement. Another atypical functional feature of PPK5, which to our knowledge has not been reported for CDKs from other organisms, is its ability to autophosphorylate in the presence of a cyclin (10). Pfmrk, a protein kinase with maximal homology to CDK-activating kinases (CAKs) of the CDK7 family (11), can also be activated by Pfcyc-1 (12). The genome of *P. falciparum* encodes at least four additional protein kinases related to CDKs (8), suggesting that additional cyclins are present as well (in eukaryotes whose genome sequence is known, the number of cyclins is larger than that of CDK catalytic subunits). PCR-based approaches have failed in the identification of malarial

cyclins (but not of CDK-related kinases), presumably because of the low level of conservation of *Plasmodium* cyclins with their counterparts in other eukaryotes. Indeed, Pfcyc-1 identification had to await the *in silico* search possibilities offered by the availability of genomic data bases (10).

In this report, we describe three novel *P. falciparum* open reading frames (ORFs) identified in the PlasmoDB data base on the basis of their relatedness to cyclins at the primary structure level. We demonstrate that these ORFs encode proteins whose abundance fluctuate during schizogony and that associate with protein kinase activity in parasite extracts, and we show that one of them is able to activate PPK5 *in vitro*. Furthermore, we demonstrate that PPK5 can be efficiently activated by RINGO, a *Xenopus* protein unrelated to cyclins but able to activate a subset of mammalian CDKs (13, 14), pointing to the possibility that cryptic cyclin-like protein may be present in malaria parasites.

## EXPERIMENTAL PROCEDURES

### Molecular Cloning and Bacterial Expression

**Pfcyc-2**—Oligonucleotides were designed for amplification of a 1173-bp region (positions 2956–4129, carrying the cyclin-related sequence) from the 6846-bp ORF (gene PFL1330c in PlasmoDB). The primers contained *Bam*HI (forward primer, GGGGGATCCATGAATGTGGTGATTTGGTG) or *Sal*I (reverse primer, GGGGTCGACAATAGTTGTATCATTTGTGTGGTC) restriction sites (single underline). After amplification from a *P. falciparum* cDNA library (kindly provided by A. Craig) with *Taq* DNA polymerase (Takara), the PCR product was digested with *Bam*HI and *Sal*I prior to insertion in the pGEX-4T3 vector (Amersham Biosciences). The pGEX-4T3-Pfcyc-2 plasmid was electroporated into *Escherichia coli* BL21, and (for all three ORF discussed here) the insert was verified by sequencing prior to expression of the recombinant protein.

Protein expression and purification were performed essentially as previously described for GST-Pfcyc-1 (10). Briefly, expression was induced for 4 h at 30 °C with 0.3 mM isopropyl-β-D-galactoside, after the 250-ml culture reached an A<sub>600</sub> value of 0.6 in 2× YT medium with 100 μg/ml ampicillin. All of the purification steps were performed at 4 °C. The bacterial pellets were lysed with lysosome and by sonication in 5 ml of lysis buffer (phosphate-buffered saline, pH 7.5, 0.1% Triton, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and Complex™ mixture protease inhibitor tablet from Roche Applied Science). The lysates were cleared by centrifugation (11000 rpm at 4 °C for 30 min), and the soluble fraction was incubated for 1.5 h at 4 °C under mild agitation with 0.5 ml of glutathione-agarose resin (Amersham Biosciences). The slurry was washed in lysis buffer, and the GST-Pfcyc-2 fusion protein was eluted with elution buffer (30 mM Tris, pH 8.0, 15 mM NaCl, 2 mM glutathione).

**Pfcyc-3**—The entire ORF (PFE0920c in PlasmoDB) was cloned into the *Bam*HI and *Sal*I restriction sites of the pMALc2x vector (New England Biolabs) after amplification from *P. falciparum* 3D7 genomic DNA using the following primers (start and stop codons in double underline): GGGGGATCCATGGATTACGAGGTAGAGGGA (forward) and GGGGTCGACTTATTTATTTCCATGTTGATC (reverse). *E. coli* DH5α cells were transformed with pMALc2x-Pfcyc-3 plasmid by electroporation. Protein expression and purification was as described above for the GST fusion proteins, except that lysis buffer was 20 mM Tris, pH 7.5, 200 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and Complex™ protease inhibitors and that the MBP-Pfcyc3 fusion protein was eluted from amylose resin (New England Biolabs) with lysis buffer supplemented with 10 mM maltose.

**Pfcyc-4**—(gene PF13\_0022 in PlasmoDB) was amplified from a cDNA library and cloned into the *Bam*HI and *Sal*I restriction sites of pGEX-4T3. Two constructs were made: the first one contained only the first exon (residues 1–75) as found in a previous version of PlasmoDB and was obtained by amplification from 3D7 (15) genomic DNA using the primers GGGGGATCCATGATGAATGACATAGTTTTA (forward) and GGGGTCGACTTATTTTACGTAAACGTCAAAATC (reverse). The second construct contained the entire PF13\_0022 ORF (5 exons), which was obtained by amplification from cDNA using the same forward primer and ACGCGTCGACCTATATACCTATTTTGTAAAGCTCCAAT-ATTC (reverse). Recombinant protein expression and purification were performed as described above for GST-Pfcyc-2. Expression and

<sup>1</sup> The abbreviations used are: CDK, cyclin-dependent protein kinase; CAK, CDK-activating kinase; ORF, open reading frame; GST, glutathione S-transferase; RT, reverse transcription; MBP, maltose-binding protein; CTD, C-terminal domain.



TABLE I  
Characteristics of the cyclin-related proteins included in this study

	PlasmoDB identifier	Pfam PF00134 score	Size (amino acids)	Maximal homology	Expected molecular mass
					<i>kDa</i>
Pfcyc-1	PF14_0605	2.2	327 (1 exon)	Cyclin H (20% identity)	39.2
Pfcyc-2	PFL1330c	8	2281 (2 exons)	Plant mitotic cyclins (13% identity)	273.5
Pfcyc-3	PFE0920c	3.2	229 (1 exon)	<i>T. brucei</i> CYC2 (26% identity)	26.8
Pfcyc-4	PF13_0022	13.4	226 (5 exons)	ania-type cyclins (33% identity)	31.1

purification of MBP-RINGO (ls26 clone) were performed as described elsewhere (13).

#### Parasite Extracts and Pull-down Experiments

*P. falciparum* (3D7) pellets were sonicated in RIPA buffer (30 mM Tris, pH 8.0, 150 mM NaCl, 20 mM MgCl<sub>2</sub>, 1 mM EDTA, 1 mM dithiothreitol, 10  $\mu$ M ATP, 0.5% Triton X-100, 1% Nonidet P-40, 10 mM  $\beta$ -glycerophosphate, 10 mM NaF, 0.1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 mM benzamide, and Complex<sup>TM</sup> protease inhibitors). The lysates were cleared by centrifugation (15,000 rpm for 15 min at 4 °C), and the total amount of proteins in the supernatant was measured by a Bio-Rad protein assay. The beads coated with GST, GST-Pfcyc-1, GST-Pfcyc-2, or GST-Pfcyc-4 were incubated in these parasite extracts or in RIPA buffer alone at 4 °C, under mild agitation for 90 min (100  $\mu$ g of total parasite proteins for 10  $\mu$ g of recombinant protein on beads). The beads were then washed three times in RIPA buffer, once in RIPA buffer with 0.1% SDS, and once in kinase buffer (25 mM Tris, pH 7.5, 15 mM MgCl<sub>2</sub>, 2 mM MnCl<sub>2</sub>, 10 mM NaF, 10 mM  $\beta$ -glycerophosphate). A kinase assay was then performed by adding 15  $\mu$ M ATP, 5  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP (3000 Ci/mmol; Amersham Biosciences), and 5  $\mu$ g of histone H1 in kinase buffer, and the samples were analyzed by SDS-PAGE and autoradiography.

#### RT-PCR

Total RNA samples were extracted from asexual cultures of clones 3D7 (15) and F12 (16) and from Percoll purified 3D7 gametocytes (stages III, IV, and V) using the Qiagen RNeasy<sup>®</sup> Mini Kit and then treated with Promega RQ1 RNase-free DNase. RT-PCRs were performed with 100 ng of total RNA using the Invitrogen SuperScript<sup>TM</sup> One-Step<sup>TM</sup> RT-PCR system with 2 mM MgSO<sub>4</sub> and 20 pmol of primers/reaction, with exception for *Pfcyc-1*, where 50 pmol of primers, and TaKaRa Ex Taq Polymerase were utilized. In control reactions reverse transcriptase was omitted, and only AmpliTaq Polymerase (TaKaRa for *Pfcyc-1*) was present. The reactions were incubated at 48 °C for 1 h; 94 °C for 2 min; 30 cycles at 94 °C for 30 s, 5 °C for 1 min, and 68 °C for 2 min; followed by 7 min at 68 °C.

The sequences of oligonucleotides specific for *Pfcyc-1*, *Pfcyc-2*, and *Pfcyc-3* are indicated above. Primers specific for genes MSP-1 and Pfg377 were as follows: MSP-1 dir, 5'-GAAGCTTTAGAAGATGCAGT-ATTGACAGG-3'; MSP-1 rev, 5'-GATATTTGAGTCTTTAATAGTGA-ACAAG-3'; Pfg377 dir, 5'-TTATCAAACGATGATACTAATGAAAATG-3'; and Pfg377 rev, 5'-ATATTTAAATTCGAAAAGTCATAG-GTAC-3'.

#### Antibodies

The following peptides were obtained from NeoSystems: *Pfcyc-1*, residues 312–326, (C)-KEDNVTEPNKKLQVS-COOH; *Pfcyc-2*, residues 376–391 of the cloned portion of the ORF, (C)-EIYSEQKYDHT-NDTTI-C-terminal amide; *Pfcyc-3*, residues 51–66, SLKNYTERIGKY-IGCS (C-terminal amide); and *Pfcyc-4*, residues 1–15, MMNDIVLINK-KKTPS-(C) (C-terminal amide). The peptides were designed with a terminal cysteine residue (except for *Pfcyc-3*, whose chosen peptide contains an internal cysteine) and coupled to rabbit albumin using 3-maleimidobenzoic acid *N*-hydroxysuccinimide ester (17). Two chickens were immunized intramuscularly in the breast muscle with 200  $\mu$ g of the peptide (as a conjugate) emulsified with Freund's complete adjuvant on day 0 of the experiment and at weeks 2, 4, and 6 with conjugate emulsified with Freund's incomplete adjuvant (17). Eggs were collected and labeled for 16 weeks after immunization and stored at 4 °C until processed. IgY was isolated from chicken yolks using the method of Polson *et al.* (18) involving a series of polyethylene glycol precipitations. The final IgY pellet was resuspended in 0.1 M sodium phosphate buffer, pH 7.6, containing 0.02% sodium azide and stored at 4 °C. IgY was isolated from eggs each week. Anti-peptide antibodies were affinity-purified by cycling the isolated antibodies over a SulfoLink-peptide affinity column prepared according to the manufac-

turer's instructions (Pierce). The antibodies were eluted with glycine HCl, pH 2.8, and the pH of the eluant was neutralized with 10% volume of 1 M phosphate buffer at pH 8.5. Protein was measured at 280 nm, and the concentration was determined using the IgY extinction coefficient of 1.25.

#### Western Blotting and Immunoprecipitation

*P. falciparum* 3D7 maintained in a modified erythrocyte culture (19) were synchronized by 5% sorbitol (20). The parasites were released from infected erythrocytes by saponin (0.1% w/v) lysis. Cell-free extracts were prepared by resuspending synchronized parasite pellets from different developmental stages of growth in M-PER Mammalian Protein Extraction Reagent, (Pierce) containing a protease inhibitor mixture (Roche Applied Science). Following 15 min of incubation at 4 °C, the lysate was centrifuged at 12,000  $\times g$  for 15 min at 4 °C. Western blot analysis was performed using Supersignal West pico kit according to the manufacturer's recommendations, using appropriate immunopurified primary and secondary antibodies. The relative intensities of the bands were quantified in a Bio-Rad Gel Doc 2000 system using Quantity One software.

For immunoprecipitation, parasite extract (100  $\mu$ g) was incubated with immunopurified anti-*Pfcyc-1*, -*Pfcyc-2*, -*Pfcyc-3*, or -*Pfcyc-4* chicken antibodies (1.5  $\mu$ g) on ice for 2 h. Protein A-Sepharose CL4B (Amersham Biosciences) was coated with anti-chicken IgY rabbit antibodies (Pierce) for 90 min under mild agitation at 4 °C in RIPA buffer and washed four times with RIPA buffer. The immunocomplexes in the parasite extract were then precipitated with 10  $\mu$ l of protein A/anti-chicken rabbit antibody beads at 4 °C under mild agitation for 90 min. The following chicken IgY antibodies were used as negative controls: nonimmune, immunopurified anti-human C5a receptor (against the peptide sequence DSKTFTPTSDTSPRKSQAV) and immunopurified anti-MIE-2, a *Trypanosoma* glucose transporter (against the peptide sequence MIEGSRNSSAVKKRLVAKPLEEC). Another negative control was performed by omitting the primary IgY altogether and incubating the extract only with anti-chicken/protein A beads. Immunocomplexes were washed three times in RIPA buffer, once in RIPA buffer with 0.1% SDS, and once in kinase buffer and finally suspended in 30  $\mu$ l of kinase buffer. The kinase assays were performed in a standard reaction as described below.

#### Kinase Assays

The assays were performed in a standard reaction (30  $\mu$ l) containing 25 mM Tris-HCl, pH 7.5, 15 mM MgCl<sub>2</sub>, 2 mM MnCl<sub>2</sub>, 15  $\mu$ M ATP, 5  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP (3000 Ci/mmol; Amersham Biosciences) and 5  $\mu$ g of histone H1 (Invitrogen) or 2  $\mu$ g GST-CTD. The reactions were initiated by addition of 0.5 or 1.0  $\mu$ g each of the recombinant protein kinase and a cyclin partner, after both proteins had been allowed to form a complex at 30 °C for 15 min in kinase assay buffer. The kinase reactions proceeded for 30 min at 30 °C and were stopped by the addition of Laemmli buffer, boiled for 3 min and analyzed by electrophoresis on 12% SDS-polyacrylamide gel. The gels were dried and submitted to autoradiography.

#### RESULTS

##### *In Silico Identification of Three P. falciparum ORFs Related to Cyclins*

We performed BLASTP searches of the PlasmoDB data base using as queries a variety of cyclin sequences from diverse organisms and also used the "text search" tool on the PlasmoDB website, using the word "cyclin" as a query. This enabled us to identify the previously characterized *Pfcyc-1* gene (10), as well as three additional ORFs, all of which displayed maximal homology to various cyclins upon BLASTP analysis of generalist data bases (Table I).

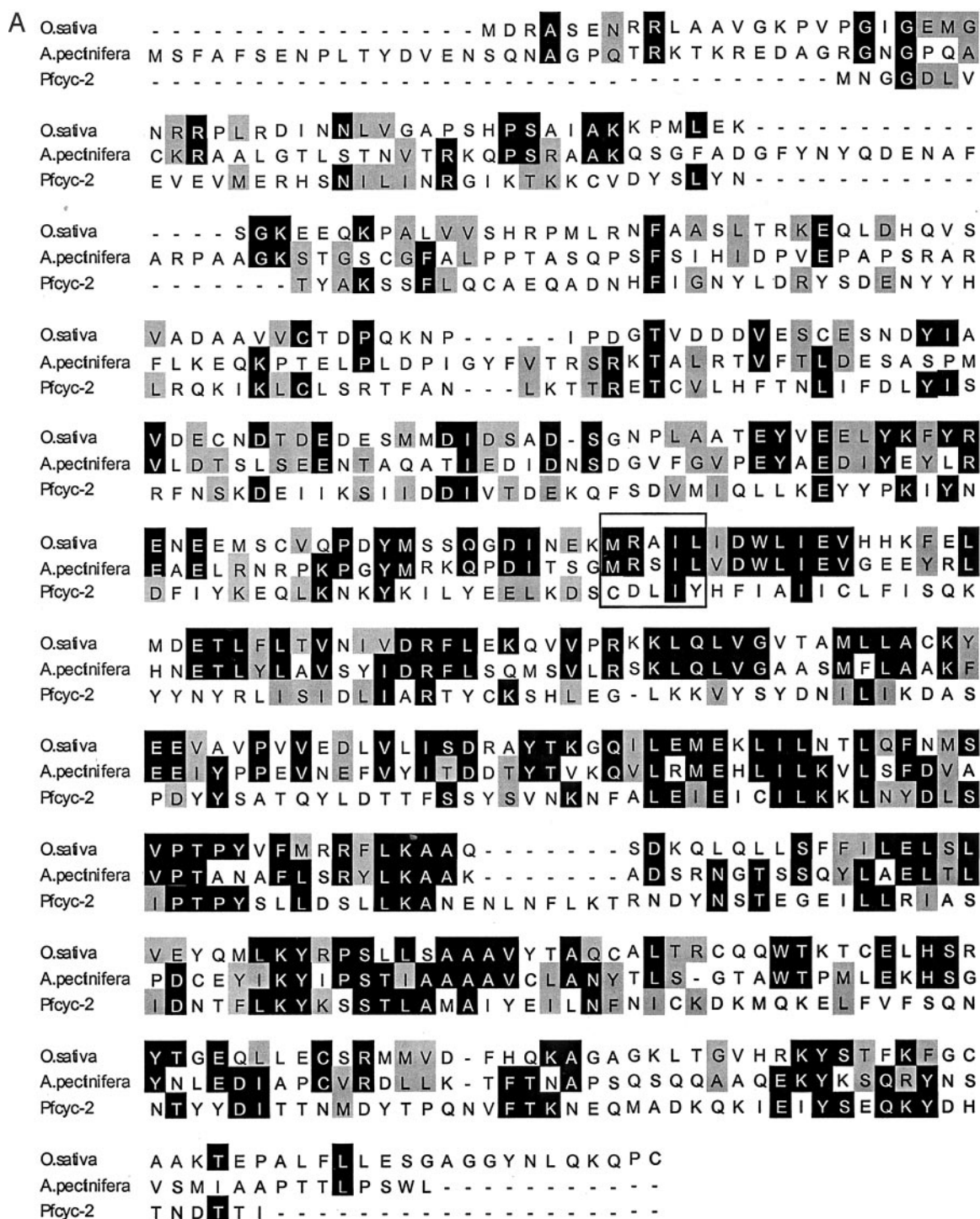


FIG. 1. Alignments of the deduced amino acid sequences of the malarial cyclins. The cyclin boxes are underlined. A, *Pfycyc-2* aligned with *Oryza sativa* CYC2B (sptr accession number Q9SSZ5) (42) and cyclin A from the starfish *A. pectinifera* (P90681). The MRAL motif cited in the text is boxed. B, *Pfycyc-3* aligned with *T. brucei* CYC2 (Q9NG22) (21) and *Trypanosoma cruzi* CYC6 (Q9GQL7) (43). C, *Pfycyc-4* aligned with a putative cyclin from *Arabidopsis thaliana* (Q94L33) and ania-6a cyclin from *Anopheles gambiae* (NCBI accession number EAA00916). The position of the frameshift mutation in the amplified fragment is indicated by a triangle.

*Pfycyc-2*—The first novel ORF we identified in this search is located on chromosome 12 (gene PFL1330c in PlasmoDB), comprises two exons, and potentially encodes a large protein; the Pf annotation predicts a 2281-residue polypeptide, which includes a 390-amino acid sequence (residues 986–1376) that meets the criteria for recognition as a cyclin in the Pfam conserved domain data base (Table I). Indeed, performing a BLASTP analysis using this region as a query to screen generalist data bases, we found that all of the high score entries were cyclins

from a variety of organisms, most of them mitotic (A and B type) cyclins. Despite this consistency, the homology is rather low (13% identity over the 390-residue cyclin-like region with a cyclin from *Asterina pectinifera*, increasing to 40% identity over the most conserved 80 residues within the cyclin box). An alignment of this region with two of the most closely related cyclins is shown in Fig. 1A.

To verify that the *Pfycyc-2* cyclin-like region was part of a much larger polypeptide, as predicted by the algorithms in



B	T. brucei	M T I M M D G P K S S T R A T E T I N Q S D G Q H R Q Q W C D R Q Q T G R N C N
	T. cruzi	- - - - - - - M S R A R A H D T Q V H V S S E M K Q L L R - R Q K - - - - -
	Pfycyc-3	- - - - - - - - - - - M D Y E V E G E V P R T D E N - - - - -
	T. brucei	S A G S D S Q S A D P Q Q S G L H E L G Q L T A V A V E R R C V E Q R H L E H L
	T. cruzi	- - - - - - - - - - - I H E L A Q L A A M V L Q E R C S Q Q R H L E Y V
	Pfycyc-3	- - - - - - - - - - - Y I F L Y L P I V L N E M I K M N N R G V G
	T. brucei	Y Q S A F H S S H V P A I S V W N Y M R R I G K Y S R C S P E C F I I C I I F I
	T. cruzi	C N T S F G S S Q I P Q I S V F D Y I R R I A K Y S Y C S P E C F V L S I I Y I
	Pfycyc-3	K I T S F H A S K V P D I S L K N Y T E R I G K Y I G C S N E C F V L L I I Y L
	T. brucei	D R Y V A A T N - C P I T F R N I H R L L I T S M L V S V K L R D D S F Y S N S
	T. cruzi	D R Y L F A T K - F P L T F R N V H R L M I T A V I V S A K L R D D T H Y S N T
	Pfycyc-3	D R L I K I H K D I S L S L L C I H R L V I T A A M I S V K F F D D L Y Y S N S
	T. brucei	Y F A G I G G V S N E E L N R L E I E F L M T I D W R T W V E P S D F N M Y C -
	T. cruzi	Y Y A S L G G I S T A E L N G L E L E F L K T I D W M T W V E P S Q F E E Y R -
	Pfycyc-3	Y Y A K I G G V T T K E L N K L E I Y F L N L I D Y K L F V S S Q E Y D F Y R K
	T. brucei	- - - - - - - - - - - - - - - - - E Q L R S R - - - - -
	T. cruzi	- - - - - - - - - - - - - - - - - A G L Q T R Y L E C Q L
	Pfycyc-3	Y I C L A V Q K Y I Y N K N N I K H I P I I K K P Y N L F N Y K S S N N S T L M
	T. brucei	C S A N Q E Q - - - - - - - - - - - - - - - - -
	T. cruzi	F S V S D E N S E Y G T S S T G G E R D T P Q - - - - - - - - - - -
	Pfycyc-3	F Q P N N N N M N I V N F A A T N Q N K K N N S Q K D Q H G N K
C	Pfycyc-4	- - - - - - - - - - - - - - - - - M M N D I V L I N K -
	A. gambiae	S A T G K A A S A V I G T T Q S T P A T A A A P Q P V Q R P Y G K I V L T L E N
	A. thaliana	- - - - - - - - - - - - - - - - - M I Y T A I D N F Y L T D E Q
	Pfycyc-4	- - - - - - - - K K T P S E E K N I D K N E E I K L R I Y G C Q L L Q E A G I I
	A. gambiae	C L L P E V K L D Q T P S Q N D G L D R E T E T D L R I L G C E L I Q T A G I L
	A. thaliana	- - - - - - - - L K A S P S R K D G I D E T T E I S L R I Y G C D L I Q E G G I L
	Pfycyc-4	L K L K A V T I V T S Q I L F H R F Y F K K S F T D F D V N I I A P S A L Y L S
	A. gambiae	L K L P Q V A M A T G Q V L F Q R F F Y S K S F V R H S M E A T A M S C I C L A
	A. thaliana	L K L P Q A V M A T G Q V L F Q R F Y C K K S L A K F D V K I V A A S C V W L A
	Pfycyc-4	C K L E E D F C R I Y K I I N T F H F L C K Y E N I K S K H I Y F D I K N L N P
	A. gambiae	S K I E E A P R R I R D V I N V F H H I K Q V R S Q K - - - - - - - - - -
	A. thaliana	S K L E E N P K K A R Q V I I V F H R M E C R R E N L P - - - - - - - - - -
	Pfycyc-4	E H F R I N I E S E E Y K N M K V D I Y T Y E L L I L K E I G F L V H K I N Q H
	A. gambiae	- P L L P M I L D Q H Y I N L K S Q V I K A E R R V L K E L G F C V H V K - - H
	A. thaliana	- L E H L D M Y A K K F S E L K V E L S R T E R H I L K E M G F V C H V E - - H
	Pfycyc-4	P H S F L L P Y I H S L F N N L N T I H K D L T K K L A Q M S W G F L N D S M R
	A. gambiae	P H K L I V M Y L K - - - - - Y L E L E K H Q N M M Q M A W N F M N D S F R
	A. thaliana	P H K F I S N Y L A T - - - - - - - L E T P P E L R Q E A W N L A N D S L R
	Pfycyc-4	T T L C C E Y Q P R C I A V A S I F L A A Y K L N I P L M N N T N W F K L F D V
	A. gambiae	T D V F V R Y Q P E T I A C A C I Y L T A R K H N I P L P N N P P W F V I F R V
	A. thaliana	T T L C V R F R S E V V A C G V V Y A A A R R F Q V P L P E N P P W W K A F D A
	Pfycyc-4	E Y E D I K K I C I R I L E L Y K I G I - - - - - - - - - - - - - - - -
	A. gambiae	S E D D M L D V C Y R I M A L Y K R G K P N A E L L E E A V E A L K K N S A N S
	A. thaliana	D K S S I D E V C R V L A H L Y S L P K A Q Y I S V C K D G K P F T F S S R S G
	Pfycyc-4	- -
	A. gambiae	G T G G G T A A P S S G S N A N S T A G L N N N E P D T A G G G T V G D G K K R
	A. thaliana	N S Q G - - - - - Q S A T K D L L P G A G E A V D T K C T A G S A N N D L K D
	Pfycyc-4	- -
	A. gambiae	S S R S R S K S L S K S S R S K S R S L S R S V S R S R S R G S R S R S R T S Q
	A. thaliana	G M V T T P H E K A T D S K K S G T E S N S Q P I V G D S S Y E R S K V G D R E
	Pfycyc-4	- -
	A. gambiae	S R S R S K T R T S R S R S R S P A S R S R S R S R S P H S P H Y S T D K S G G
	A. thaliana	R E S D R E K E R G R E R D R G R S H R G R D S D R D S D R - - - - -
	Pfycyc-4	- -
	A. gambiae	G G G G T R R T S K K T R H R S R S P A K V S S S G K K K S S R H Y S S R S P S
	A. thaliana	- -
	Pfycyc-4	- -
	A. gambiae	P D S P Q K Y K K S R H R S S K H E R D R S R D R D R D R R R
	A. thaliana	K D S G G H S D K S R H H S S R D R D Y R D S S K D R R R H H

FIG. 1—continued

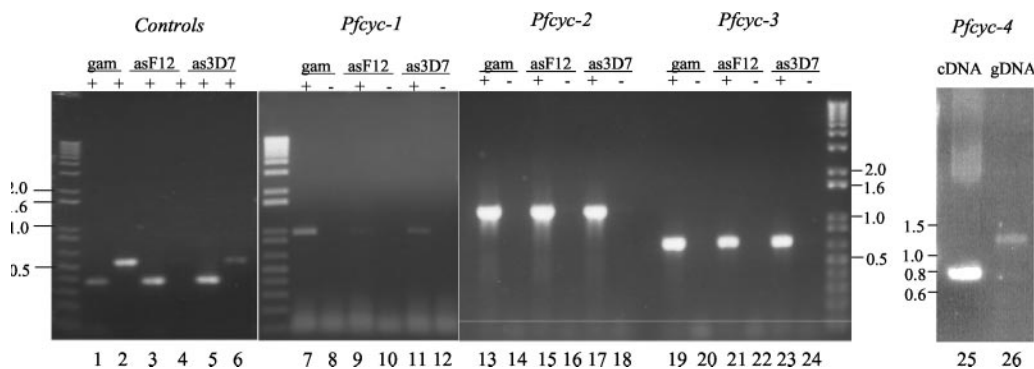


FIG. 2. RT-PCR analysis of *Pfycyc-1* (lanes 7–12), *Pfycyc-2* (lanes 13–18), and *Pfycyc-3* (lanes 19–24) using RNA from gametocytes (*gam*) or asynchronous asexual stages (*as*) from *P. falciparum* clones 3D7 or F12. The plus and minus signs indicate RT-PCRs performed in the presence or absence of reverse transcriptase, respectively. The controls to the left concern RT-PCR with primers to the following genes. Lanes 1, 3, and 5, *msp-1*; lanes 2, 4, and 6, *pfg377*. The right panel shows *Pfycyc-4* PCR products obtained from a cDNA library from asexual stages (lane 25) and from genomic DNA (lane 26).

PlasmoDB, we performed RT-PCR experiments (data not shown) with *P. falciparum* mRNA and primers designed from the predicted ends of the entire ORF. This enabled us to amplify the entire region (2.7 kb) lying 3' to the cyclin-related sequence. Sequence analysis demonstrated that the 5' boundary of the intron is shorter than that predicted in PlasmoDB; in our cDNA the intron was located between positions 6170 and 6327 (counting from the initiation codon) instead of positions 6104 and 6327 as predicted in the data base. We were unable to obtain PCR product starting at the predicted translation initiation site, presumably because of the large size of the mRNA. However, we obtained a PCR product extending 1.3 kp upstream of the cyclin-related region. Taken together, these experiments demonstrate that the *Pfycyc-2* mRNA is at least 5.2 kb in size (from nucleotides 1633 to 6846 in the PlasmoDB entry). Furthermore, sequence analysis showed that a continuous ORF embeds the cyclin-related region, as predicted on PlasmoDB (although we noticed a 1-base deletion in a stretch of As outside the cyclin region (position 4376), probably arising from polymerase slippage during reverse transcription; this is a common occurrence with the AT-rich malarial genes).

*Pfycyc-3*—This single-exon ORF is located on chromosome 5 (gene PFE0920c) and potentially encodes a 229-amino acid polypeptide. The ORF is flanked on both sides by regions that are extremely AT-rich, which are characteristic of noncoding DNA in *P. falciparum*. The highest score obtained by BLASTP analysis of the *Pfycyc-3* ORF on generalist data bases corresponds to the *Trypanosoma brucei* CYC2 gene, which was identified by complementation of a yeast G<sub>1</sub> cyclin mutant (21). *Pfycyc-3* shows 26% identity to TbCYC2 over the entire ORF and 44% identity over the most conserved region (residues 40–155; see Fig. 1B for an alignment). Most other BLASTP high score entries are cyclin-related proteins.

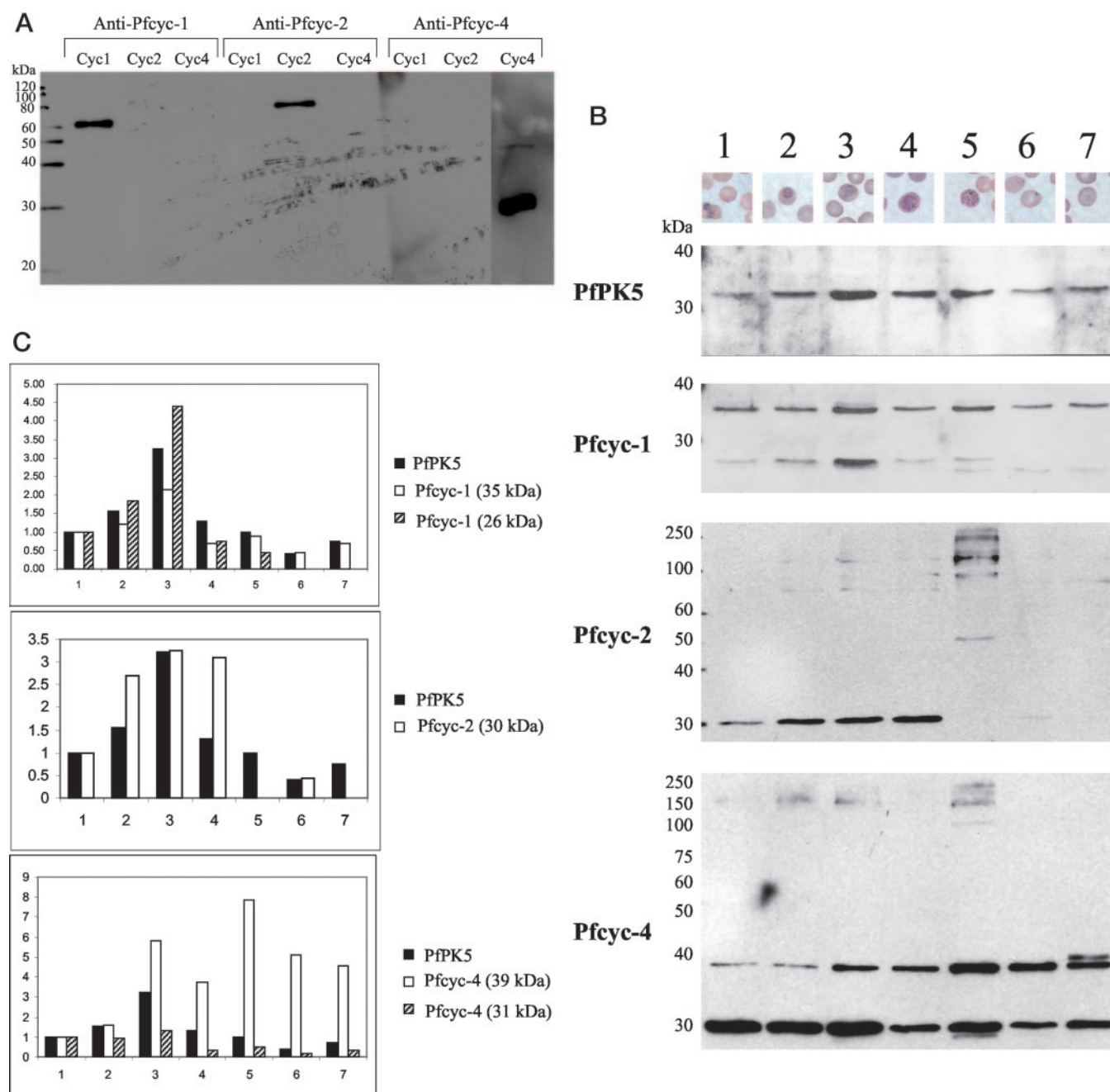
*Pfycyc-4*—The last cyclin-related sequence identified in this search was initially found in PlasmoDB as a 74-amino acid ORF located on chromosome 13. Subsequent experimental data from Western blot experiments (see below) suggested that the ORF should actually be larger, and we therefore re-examined the latest version of PlasmoDB. Indeed, the *Pfycyc-4* ORF had been updated, and now gene prediction algorithms all predicted ORFs between 202 and 262 amino acids, the discrepancies being due to differences in the number and boundaries of predicted introns. RT-PCR using primers designed to discriminate between the three models, followed by sequence analysis of the amplicons, allowed us to establish the validity of the 5-exon Pf annotation prediction (gene PF13\_0022). The 262-residue, 31-kDa polypeptide potentially encoded by the *Pfycyc-4* ORF is

recognized as a cyclin by the SMART conserved domain data base (entry: smart00385.5) and contains two cyclin boxes, like the recently described ania-6-type cyclins (22), to which it appears to be related. Interestingly, the highest BLASTP scores for the entire *Pfycyc-4* ORF correspond to uncharacterized ania-like cyclins from insects (33% identity with an *Anopheles* cyclin), followed by ania cyclins from plants and mammals (see Fig. 1C for an alignment). Surprisingly, all amplicons obtained from several independent RT-PCR experiments had a frame-shift mutation (deletion of one base in a stretch of 7 As) at position 565, resulting in a predicted polypeptide containing one cyclin box instead of two (see Fig. 1C). As will be shown below, the N-terminal part of the protein is sufficient to display protein-kinase binding properties.

#### Expression of the Cyclin-related Genes in Erythrocytic Stages

*mRNAs*—To determine whether the newly found genes are expressed during erythrocytic infection, we performed RT-PCR experiments on total RNA from *in vitro* cultures of two clones, the reference clone 3D7 (15) and its gametocyteless derivative F12 (16), and from Percoll purified 3D7 gametocytes (Fig. 2). RT-PCRs specific for the genes *MSP-1*, encoding the merozoite-specific surface antigen 1 (23), and *pfg377*, encoding the gametocyte-specific protein Pfg377 (24), were used to characterize the RNA samples prior to cyclin expression analysis. The *msp-1*-specific band indicated, as expected, the presence of schizont RNA in both asexual cultures of 3D7 and F12 and detected the unavoidable schizont contamination (~4% by morphological examination) in the sample of Percoll-purified gametocytes. The *pfg377* specific band, readily detectable in RT-PCR from gametocyte RNA, was detectable at a much lower level also in the 3D7 asexual RNA, diagnostic of the presence of a minor gametocyte fraction. No *pfg377* amplification band was present in RT-PCR from RNA from F12, a gametocyteless clone. RT-PCR of *Pfycyc-2* and *Pfycyc-3* yielded strong signals both from RNA from asexual stages and from gametocytes. In contrast, the *Pfycyc-1* amplification product was much more abundant when gametocyte RNA was used than in the asexual samples, suggesting that this gene may be developmentally regulated and predominantly expressed in sexual stages (although *Pfycyc-1* protein is readily detected in asexual parasite protein extracts; see below). In all cases, negative controls in which reverse transcriptase had been omitted gave no signal, allowing us to exclude the possibility that amplification bands derived from contaminating genomic DNA. At the time we performed this series of RT-PCR experiments, *Pfycyc-4*-specific





**FIG. 3. Western blot analysis.** *A*, specificity of the immunopurified anti-Pfcyc-1, -2, and -4 antibodies. Membranes carrying recombinant GST-Pfcyc-1, GST-Pfcyc-2, and GST-Pfcyc4/CB were probed with the antibodies indicated above. This demonstrates the absence of cross-reactivity of these antibodies with the noncognate cyclins. *B*, Western blot analysis of stage specific *P. falciparum* extracts. 20  $\mu$ g of synchronized stage specific extracts obtained at 6-h intervals starting from 18 h post-sorbitol treatment were resolved in 12% SDS-polyacrylamide gels. Mouse PfPK5 (1:500) antibody and chicken Pfcyc-1 (1:750), Pfcyc-2 (1:2,000), and Pfcyc-4 (1:10,000) antibodies were used with appropriate secondary antibodies. A representative parasite infected erythrocyte from each time point is shown in the top panel. Lanes 1–3, trophozoite; lanes 4 and 5, schizont; lanes 6 and 7, ring. *C*, quantification of the Western blot signals. The densitometric value of each band was normalized against the total amount of protein in each lane, as determined by Coomassie staining/densitometric measurement of two gels loaded with identical volumes of each extract. The relative intensities are given as the ratios to the value of the relevant band in lane 1. For comparative purposes, the values for PfPK5 are given in relation to those for the three cyclins.

oligonucleotides were not available. We were however able to demonstrate that an amplification product corresponding to the spliced transcript was readily obtained from a blood stages cDNA library, suggesting that the gene is expressed in these stages (Fig. 2). This was confirmed by Western blot analysis (see below).

**Proteins**—We next obtained peptide-derived antibodies against the polypeptides encoded by the four cyclin-related genes. The antibodies against peptides derived from Pfcyc-1, Pfcyc-2, and Pfcyc-4 recognized their cognate recombinant pro-

tein and showed no cross-reactivity with noncognate cyclin-like proteins (Fig. 3A). Antibodies directed against Pfcyc-3 failed to produce a signal on Western blots (but see below for immunoprecipitation results). Western blot analysis of synchronized parasites (Fig. 3, B and C) confirmed that Pfcyc-1, Pfcyc-2, and Pfcyc-4 are expressed in asexual intraerythrocytic parasites and indicated that the abundance of all three gene products fluctuates during progression of the schizogonic cycle. Pfcyc-1 appears as a 37-kDa band (in good agreement with the calculated molecular mass of 39.2 kDa) whose abundance peaks in

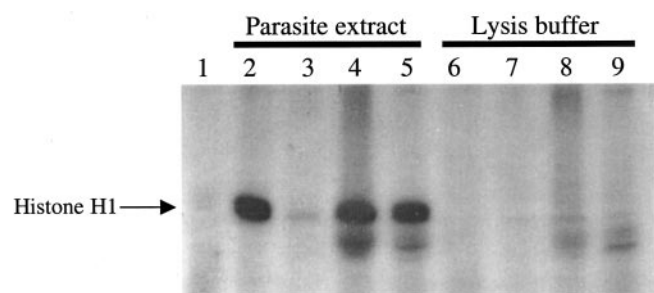


FIG. 4. Pull-down experiment using recombinant cyclins, coupled to kinase assays. GST-Pfcyc-1 (lanes 2 and 6), GST-Pfcyc-2 (lanes 3 and 7), GST-Pfcyc-4/NT (lanes 4 and 8), and GST-Pfcyc-4/CB (lanes 5 and 9) were incubated either in parasite extract (lanes 2–5) or in parasite lysis buffer without parasite material (lanes 6–9). The beads were washed, and bound material was assayed for Histone H1 kinase activity. The kinase reactions were then run on a polyacrylamide gel that was subjected to autoradiography. Lane 1 shows the background signal of a kinase assay (histone H1 only, without any malarial recombinant protein or extract).

mature trophozoites, like PfkPK5; the lower band visible on the Western blot follows a similar distribution and may represent a degradation product. The calculated molecular mass of Pfcyc-2 is 276.1 kDa. Accordingly, the Pfcyc-2 antibody reveals a set of large bands (50 to ~250 kDa) present predominantly in segmenters, although some of these are visible throughout the cycle. Interestingly, the most prominent band detected with the Pfcyc-2 antibody has an apparent size of 30 kDa and peaks in trophozoites. Whether or not this band arises from proteolytic cleavage from the large Pfcyc-2 polypeptide remains to be established (see “Discussion”). The antibody against Pfcyc-4 (calculated molecular mass, 31.1 kDa) yields two major bands with distinct expression patterns: a 31-kDa band peaks in trophozoites and a 39-kDa species that clearly shows maximum expression in segmenters, with high levels maintained in rings. As is the case for Pfcyc-2, the Pfcyc-4 antibody also detects high molecular weight bands (see “Discussion”). Competition experiments with the Pfcyc-2 and Pfcyc-4 antibodies (not shown) demonstrated that incubation of the antibodies with the relevant peptides abolished the Western blot signals.

#### Association of Recombinant Pfcyc-1 and Pfcyc-4 with Histone H1 Kinase Activity

We next wanted to investigate the biochemical properties of the proteins encoded by the Pfcyc genes, especially with respect to their potential association with kinase activity. To this end, we expressed all four proteins (Pfcyc-1 to Pfcyc-4) in *E. coli*. The entire coding regions of Pfcyc-1 and Pfcyc-3 were cloned into pGEX vectors to generate GST-cyclin fusion proteins; for the Pfcyc-2 ORF, of which only a small region is related to cyclins, we chose to express only the region between residues 986 and 1376, which spans the cyclin-like region. Two constructs were made for Pfcyc-4: the first one (Pfcyc-4/NT) encoded the 74 N-terminal residues (*i.e.* as found in our first data base search; see above) fused to GST. After identifying a larger Pfcyc-4 ORF in PlasmoDB, we prepared another plasmid (Pfcyc-4/CB) containing the entire predicted coding region (carrying a frame-shift deletion at residue 565 and therefore expressing only one full cyclin box) (see above and Fig. 1C). Expression was successful for Pfcyc-1, Pfcyc-2, Pfcyc-4/CB, and Pfcyc-4/NT; however, we were unable to express Pfcyc-3 as a GST fusion protein. We therefore switched to the expression system generating a fusion with the maltose-binding protein (MBP). This somewhat improved the yield, but the total amount obtained was not sufficient to perform pull-down experiments with this particular protein.

We performed pull-down experiments to determine whether

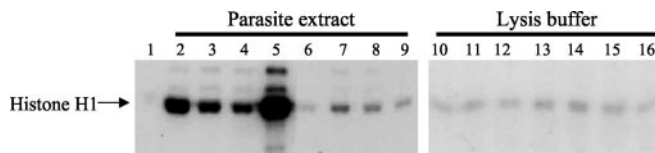
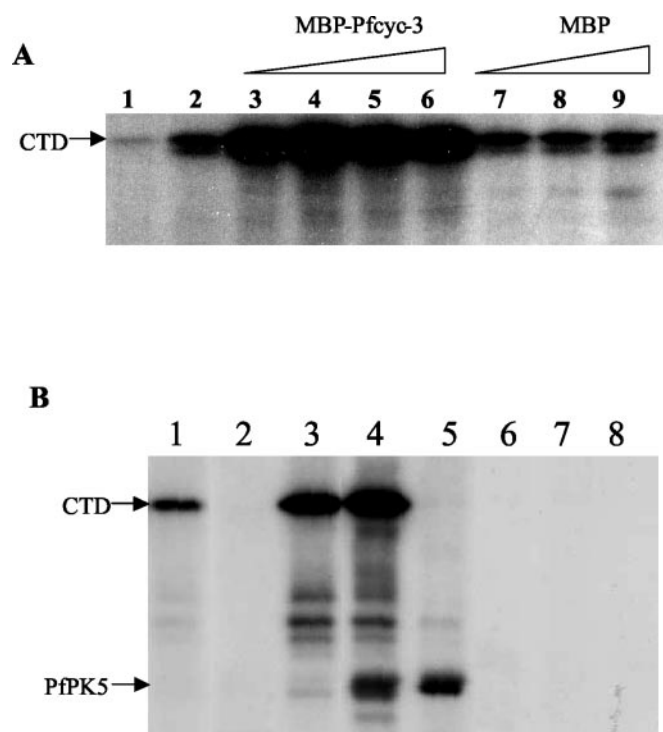


FIG. 5. Immunoprecipitations from parasite extracts using anti-cyclin antibodies, coupled to kinase assays. Immunopurified chicken IgY antibodies against peptides from Pfcyc-1 (lanes 2 and 10), Pfcyc-2 (lanes 3 and 11), Pfcyc-3 (lanes 4 and 12), and Pfcyc-4 (lanes 5 and 13) were incubated in parasite extracts (lanes 2–9) or parasite lysis buffer without parasite material (lanes 10–16). Negative controls consisted of nonimmune unpurified IgYs (lanes 6 and 14), immunopurified IgY antibodies against the C5a receptor (lanes 7 and 15) or MIE-2 (lanes 8 and 16), and immunoprecipitation performed in the absence of IgY antibodies (secondary anti-chicken IgY antibodies only) (see “Experimental Procedures”). Immune complexes were pelleted using protein A-agarose beads coated with rabbit anti-IgY secondary antibodies. The beads were washed, and bound material was assayed for Histone H1 kinase activity. The kinase reactions were then run on a polyacrylamide gel, which was subjected to autoradiography. Lane 1 shows the background signal of a kinase assay (histone H1 only, without any immunoprecipitate).

the recombinant cyclin-related molecules were able to associate with a kinase activity in parasite extracts. Glutathione-agarose beads were coated with similar amounts of either GST alone (as a negative control), GST-Pfcyc-1, GST-Pfcyc-2, GST-Pfcyc-4/NT, or GST-Pfcyc-4/CB. The beads were then incubated in either parasite extract (asynchronous asexual stages) or parasite lysis buffer alone (without parasite material) and washed. The material bound to the beads was subsequently assayed for histone H1 kinase activity (Fig. 4). No such activity was detectable in any sample that had not been in contact with the parasite protein extract (lanes 6–9), indicating that the H1 kinase activity is not of bacterial origin. In those samples that had been incubated in parasite extract, a weak signal, presumably originating from nonspecific binding of protein kinases to the beads, was present in the “GST alone” negative control (not shown). A signal of identical intensity was observed on the beads coated with GST-Pfcyc-2 (lane 3), indicating that this particular recombinant protein did not associate specifically with a kinase activity under these conditions (and, incidentally, provides a valuable additional negative control). In contrast, a strong signal was detected when the beads had been coated with Pfcyc-1 (lane 2) and Pfcyc-4 (lanes 4 and 5). In the latter case, the recombinant protein containing only the 74 N-terminal residues of Pfcyc-4 was as efficient as the protein containing a entire cyclin box to pull down the kinase activity (compare lanes 4 and 5). These data strongly suggest that the proteins encoded by the Pfcyc-1 and Pfcyc-4 genes are able to bind to a protein kinase present in the parasite extract and are consistent with the *in vitro* cyclin activity that we reported previously for Pfcyc-1 (10).

#### Immunoprecipitation of Histone H1 Kinase Activity Using Antibodies against Four *P. falciparum* Cyclin-related Proteins

The ability of the malarial cyclin-related proteins to associate with a kinase activity from parasite extracts was also investigated by immunoprecipitation of the native proteins in parasite extracts using the monospecific, immunopurified antibodies described above (Fig. 5). This allowed us to test a possible association of Pfcyc-3 (which was not produced in sufficient amounts in *E. coli* to allow us to perform the pull-down experiment described above) with kinase activity in parasite extracts. Immunoprecipitates obtained with Pfcyc-1 and Pfcyc-4 antibodies contained a high level of histone kinase activity (lanes 2 and 5), thereby independently confirming the pull-down data. Immunoprecipitated Pfcyc-2 and

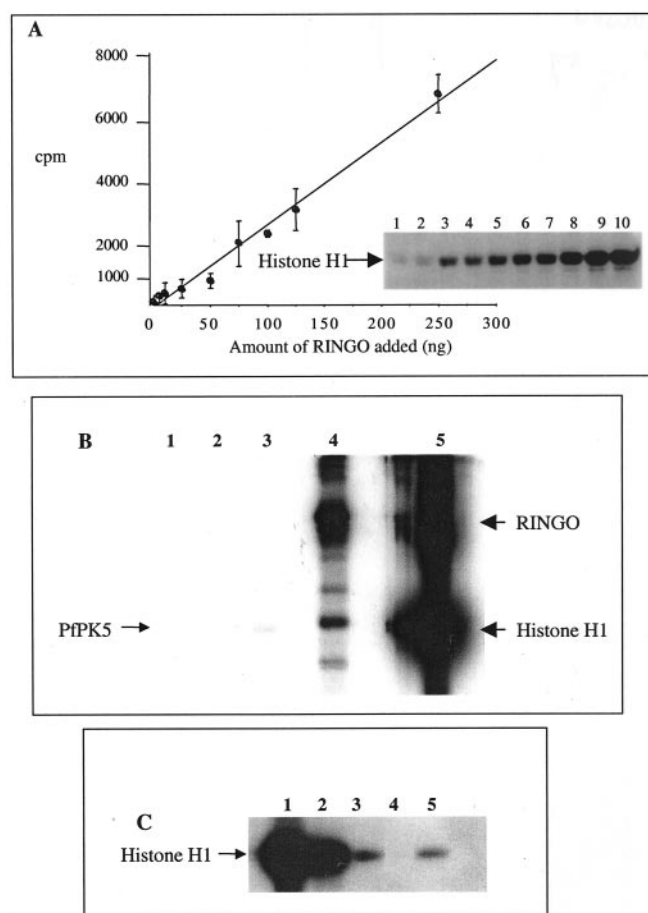


**FIG. 6. Stimulation of recombinant PfPK5 by recombinant Pf-cyc-3.** A, dose dependence. Kinase assays were performed using 2 µg of GST-CTD as a substrate, with 1.0 µg of His-PfPK5 and the following volumes of an MBP-Pf-cyc-3 preparation: lane 2, no MBP-Pf-cyc-3; lane 3, 0.4 µl; lane 4, 0.8 µl; lane 5, 1.6 µl; lane 6, 3.2 µl. The reactions for lanes 6–8 contained no MBP-Pf-cyc-3 but 0.6 µg (0.4 µl), 1.2 µg (0.8 µl), and 4.8 µg (2.0 µl) MBP moiety alone, respectively. Lane 1 shows the background signal of a kinase assay (GST-CTD only, without any other recombinant protein). B, PfPK5 autophosphorylation in the presence of GST-Pf-cyc-1 but not in the presence of MBP-Pf-cyc-3. Kinase assays were performed with the following proteins: lane 1, 1.0 µg of His-PfPK5 and GST-CTD; lane 2, GST-CTD; lane 3, GST-CTD, His-PfPK5, and MBP-Pf-cyc-3 (1 µl); lane 4, GST-CTD, His-PfPK5, 1 µg of GST-Pf-cyc-1; lane 5, His-PfPK5, GST-Pf-cyc-1; lane 6, His-PfPK5 and MBP-Pf-cyc-3; lane 7, GST-Pf-cyc-1 and GST-CTD; lane 8, MBP-Pf-cyc-3 and GST-CTD. The kinase reactions were then run on a polyacrylamide gel that was subjected to autoradiography.

Pf-cyc-3 (lanes 3 and 4, respectively) also gave signals that were repeatedly higher than background (lanes 6 and 7), albeit to a lesser extent than Pf-cyc-1 and Pf-cyc-4. This suggests that all four cyclin-related proteins associate with histone H1 kinase activity present in the parasite extract (see “Discussion”).

#### Activation of PfPK5 by Recombinant Pf-cyc-3

We demonstrated earlier that PfPK5, the malarial protein kinase that is most closely related to CDK1, displays a relaxed specificity with respect to the cyclins that can activate it *in vitro* (10). We therefore tested the ability of the three novel recombinant cyclin-related proteins to activate His-tagged PfPK5. GST-Pf-cyc-1 used as a positive control repeatedly had the previously described (10) ability to stimulate PfPK5, using as substrates either histone H1 or the C-terminal domain (CTD) of RNA polymerase II. GST-Pf-cyc-2 never displayed any significant PfPK5-activating activity in this series of experiments, whereas some batches of GST-Pf-cyc-4 displayed marginal levels of activity (data not shown). In contrast, MBP-Pf-cyc-3 stimulated PfPK5 activity against the CTD in a dose-dependent manner, whereas the MBP moiety alone showed no effect (Fig. 6A). The level of PfPK5 activation by MBP-Pf-cyc-3 was heavily dependent on the batches of recombinant proteins, with some preparations lacking activity altogether. MBP-Pf-



**FIG. 7. Stimulation of PfPK5 activity by recombinant RINGO protein.** A, dose dependence. Inset, kinase assays were performed with 1 µg of recombinant (His-tagged) PfPK5 and the following amounts of MBP-RINGO: lane 3, 5 ng; lane 4, 10 ng; lane 5, 25 ng; lane 6, 50 ng; lane 7, 75 ng; lane 8, 100 ng; lane 9, 125 ng; lane 10, 250 ng. Lane 1 is histone substrate alone (5 µg) (no PfPK5), and lane 2 shows the basal activity of PfPK5 (in the absence of RINGO). To obtain a graphical representation, similar reactions as those described above were performed and the reaction mixtures were spotted on P81 paper for quantification (see “Experimental Procedures”). Each point represents an average value from two different experiments, each done in duplicate. The standard deviation is indicated. B, possible PfPK5 autophosphorylation in the presence of MBP-RINGO. Kinase assays were performed with the following proteins: lane 1, 5 µg of histone H1; lane 2, RINGO (0.25 µg) and histone H1; lane 3, His-PfPK5 (0.5 µg) and histone H1; lane 4, His-PfPK5 and RINGO; lane 5, His-PfPK5, RINGO and histone H1. The kinase reactions were then run on a polyacrylamide gel that was subjected to autoradiography. C, comparative efficiency of MBP-RINGO and GST-Pf-cyc-1 for activation of His-PfPK5. PfPK5 (50 ng) kinase assays were performed with the following recombinant proteins, using histone H1 as a substrate: lane 1, 250 ng of MBP-RINGO; lane 2, 100 ng of MBP-RINGO; lane 3, 50 ng of MBP-RINGO; lane 4, no activator protein; lane 5, 1 µg of GST-Pf-cyc-1. Lane 6 is the negative control (reaction with histone H1 but no recombinant proteins). After electrophoresis, the gel was autoradiographed for 30 min.

cyc-3 was poorly expressed in *E. coli*, and the amounts of protein obtained were in general too low to allow quantification of the full-length fusion protein prior to performing the kinase assay (this explains why the amount of MBP-Pf-cyc-3 used in these experiments is expressed in the figure legend as the volume of purified protein rather than as the mass of recombinant protein). Therefore, it appears that the “specific activity” of MBP-Pf-cyc-3 in this assay was relatively high compared with that of GST-Pf-cyc-1 (which was routinely used in microgram amounts).

An unexpected property of PfPK5 was its ability to autophosphorylate in the presence of GST-Pf-cyc-1 (10). To determine



whether the enzyme would also display this property in the presence of MBP-Pfcyc-3, we performed kinase assays using both recombinant cyclins in such conditions as to obtain a similar level of PfPK5 activity toward CTD (Fig. 6B, compare lanes 3 and 4). It is clear that PfPK5 autophosphorylates in the presence of Pfcyc-1 (lanes 4 and 5) but not in the presence of Pfcyc-3 (lane 3 and 6).

#### Activation of PfPK5 by RINGO

The *in silico* searches that lead to the identification of Pfcyc-1 to Pfcyc-4 were based solely on homology to cyclins. However, in other systems there are examples of CDKs being activated by proteins that show no obvious sequence homology to cyclins. For example, mammalian p25, an activator of CDK5 with no relatedness to cyclins at the primary structure level, is very similar to cyclins in its tri-dimensional structure because it shares the so-called “cyclin fold”; we demonstrated earlier that p25 is an efficient activator of PfPK5 (10). Another “noncyclin” activator of CDK1 and CDK2, RINGO, has more recently been described (13, 14). As shown in Fig. 7, RINGO is an efficient activator of PfPK5. The addition of increasing amounts of recombinant RINGO (from 0 to 250 ng) to a fixed amount of PfPK5 (1  $\mu$ g) resulted in a linear increase in kinase activity (Fig. 7A, lanes 2–10). With larger amounts of RINGO, a plateau effect was observed, as expected (data not shown). Obviously, we verified (i) that the preparations of MBP-RINGO used for these experiments did not carry any intrinsic kinase activity and (ii) that the MBP moiety alone has no effect on PfPK5 activity (data not shown, but see Fig. 6A, lanes 7–9). As is the case with Pfcyc-1 but not Pfcyc-3 (Fig. 6B), PfPK5 appears to undergo autophosphorylation in the presence of RINGO (Fig. 7B, lane 4); however, because the RINGO protein itself is efficiently phosphorylated by PfPK5 (Fig. 7B, lanes 4 and 5), we cannot at this stage exclude that the band observed at 34 kDa in Fig. 7B is a RINGO degradation product rather than phosphorylated PfPK5. Although it is difficult to accurately quantify the stimulatory effect of the various activators on PfPK5 because of the very low level of basal activity of the kinase, RINGO clearly appears to be a stronger activator of the malarial CDK than other recombinant proteins (such as Pfcyc-1) tested at similar concentrations; 20-fold more GST-Pfcyc-1 than MBP-RINGO was needed to generate a similar signal from a fixed amount of PfPK5 (Fig. 7C). This is in agreement with our independent estimations of a PfPK5 activation factor of up to 3 orders of magnitude by GST-Pfcyc-1 (10) and of more than 4 orders of magnitude by MBP-RINGO (this study and data not shown).

#### DISCUSSION

**Identification of Three Novel Potential Cell Cycle Regulators in Malaria Parasites—Plasmodium** post-genomics is coming of age, and the availability of sequence data bases for several species of malaria parasites has revolutionized the study of the molecular biology of this important genus of pathogenic organisms. An obvious benefit of this recent development is the ease with which putative homologues of gene products of interest (notably with respect to potential drug or vaccine targets) can be identified, cloned, and characterized (25). Here we report the identification and preliminary characterization of three genes encoding novel putative *P. falciparum* cell cycle regulators.

The purpose of this study was to identify *P. falciparum* regulators of cyclin-dependent kinases. In addition to Pfcyc-1 (10), searches of the *P. falciparum* genomic data base has revealed three additional cyclin-related proteins, all of which appear to be expressed in blood stages of the infection. The three different molecules for which Western blot analysis was

possible, Pfcyc-1, Pfcyc-2, and Pfcyc-4, have characteristic patterns of expression, which may indicate that they have distinct and nonredundant roles in the malaria cell cycle. For example, the 30-kDa form of Pfcyc-4 is present in high levels in segmenters and in rings but in lower levels in trophozoites, in marked contrast with Pfcyc-1, which peaks in trophozoites, similar to PfPK5, a *P. falciparum* CDK1 putative homologue (8). Furthermore, these molecules appear to undergo differential processing and/or degradation processes. Differential splicing modifications cannot be ruled out as a possible explanation for the presence of some of the observed protein species. Indeed, each of the cyclin genes identified in the present study would warrant a detailed study of the structure of its mRNA(s) and of the post-translational modifications its products undergo during the life cycle of the parasite.

**Biochemical Properties of the Novel Plasmodium Cyclins—**The products of Pfcyc-1, Pfcyc-3, and Pfcyc-4 possess functional properties consistent with their belonging to the cyclin family, *i.e.* association with histone H1 kinase activity present in parasite extracts (Pfcyc-4; see Figs. 4 and 5) or direct activation of PfPK5 *in vitro* (Pfcyc-3; see Fig. 6). Although all of the Pfcyc-4 amplicons we obtained from cDNA encoded only one of the two cyclin boxes because of a frameshift deletion (Fig. 1C), the recombinant protein was able to associate to a kinase activity. Furthermore, a short region of Pfcyc-4 (the 75 N-terminal residues) is sufficient to display this kinase-binding activity. This study also shows that Pfcyc-1, for which available functional data were thus far restricted to the demonstration of PfPK5 activation *in vitro*, associates with a histone kinase activity in parasite extracts. In contrast, the recombinant cyclin-like domain contained within the (much larger) Pfcyc-2 ORF was unable to pull down a kinase activity from parasite extracts, although immunoprecipitates from such extracts obtained by using a mono-specific anti-Pfcyc-2 antibody contained levels of histone H1 kinase activity that were significantly above background. A possible explanation for this apparent discrepancy is that the domain expressed in recombinant Pfcyc-2 may lack regions that are essential for interaction with the kinase subunit. An alternative possibility is that the cognate catalytic subunit (see below), or an additional required factor, was not present in sufficient amounts in the free, unbound form in the parasite extracts to allow detection under these conditions. Although the immunoprecipitation data are consistent with a cyclin function for Pfcyc-2, we cannot formally exclude at this stage that they arose from cross-reactivity of the antibody to a different protein present in the parasite extract; although the immunopurified anti-Pfcyc-2 antibody does not show any cross-reactivity with recombinant Pfcyc-1 or Pfcyc-4 (Fig. 3A), cross-reactivity with another as yet unidentified protein with cyclin-like activity is possible (albeit unlikely). A scan of the entire *P. falciparum* genome with the sequences of the peptides used for immunization showed that these sequences are indeed unique and that no closely related sequences are found in PlasmoDB.

Notwithstanding Pfcyc-2, the present study brings to three the number of parasite proteins with demonstrated cyclin properties (Pfcyc-1, Pfcyc-3, and Pfcyc-4). Their identity was uncovered on the basis of overall homology levels, but none of these sequences possess specific motifs found in cyclins in other systems. For example, the MRAIL motif, which in many cyclins mediates interactions with the RXL motif present in substrates or regulators (26, 27), is not present in the plasmodial molecules. The “destruction box” targeting proteins (including many cyclins) for ubiquitin-dependent proteolysis (28, 29) is also apparently missing. In this respect, it is worth mentioning that malaria parasites appear to possess a ubiquitin pathway (30);

whether or not it is used to regulate cyclin levels remains to be investigated. In particular, it would be of interest to determine whether some of the high molecular mass bands observed on Western blots (Fig. 3) may be polyubiquitinated forms of the proteins.

We identified Pfcyc-3 as yet another cyclin (and the second one from *P. falciparum*, the first one being Pfcyc-1) able to activate PfPK5 *in vitro*, confirming the promiscuity of this kinase with respect to its activating partners. Obviously, the relevance of this finding remains to be established in an *in vivo* context. Interestingly, Pfcyc-1 and Pfcyc-3 have differential effects on PfPK5; in conditions where both cyclins activate the kinase to a similar level toward an exogenous substrate (Fig. 6B), it is only in the presence of Pfcyc-1 that autophosphorylation occurs (the nonplasmodial activator RINGO seems to behave like Pfcyc-1 in this respect (Fig. 7B)). Autophosphorylation has to our knowledge not been documented in CDKs from other systems, even *in vitro*. The identity of the target residue and whether or not this phenomenon has any physiological relevance remain to be determined. It is still not clear whether or not *P. falciparum* possesses a CAK; Pfmrk, a kinase with maximal homology to the mammalian CAK CDK7, can be activated *in vitro* by Pfcyc-1 and by mammalian cyclin H to phosphorylate histone H1 but does not appear to use PfPK5 as a substrate in these conditions (10). The observation that RINGO, which activates mammalian CDKs in a CAK-independent manner (14), is a powerful activator of PfPK5 may suggest that this enzyme may be regulated in a similar (CAK-independent) way *in vivo*.

**Cryptic Cell Cycle Regulators in the *P. falciparum* Genome?**—In eukaryotic systems where cell cycle control is better understood, the number of cyclins is generally larger than that of catalytic subunits, and in several instances a given catalytic subunit can be activated by a number of different cyclins, each of which confers different catalytic properties (*e.g.* in terms of substrate specificity) to the kinase subunit (31, 32). A superficial examination of the *P. falciparum* genome CDK and cyclin content, based on sequence homology, seems to suggest that the number of cyclins is surprisingly small. However, the observation that RINGO (this study) and p25 (10), two proteins with no homology to cyclins at the primary structure level, are efficient activators of a malarial CDK, together with the fact that ~50% of the ORFs in the *Plasmodium* genome are not related to any known protein (3), leaves open the possibility that “cryptic” cyclin functional homologues may be present in *Plasmodium*. It is relevant to mention here that no obvious homologues of RINGO or p25 have yet been identified in PlasmoDB.

**Reconstructing the Plasmodium Cell Cycle Control Network**—A possible avenue to overcome the problem of identification of cryptic CDK regulators is to exploit genomic data bases in a mass spectrometry-based approach, in which proteins interacting with a given tagged CDK (or cyclin) can be purified from parasite extracts by affinity chromatography and hydrolyzed into peptides. They can then be subjected to mass spectrometry analysis and identified against genomic data bases (33, 34). This should allow us not only to identify novel (possibly cryptic) functional homologues of cell cycle regulators but also to determine which already characterized molecules form cognate CDK-cyclin pairs. This presents a particular interest in the context of investigations into regulatory networks of such a phylogenetically isolated group of organisms as the Apicomplexa, where the precise function of proteins often cannot be predicted from sequence alone. For example, one would not have predicted that PfPK5, a putative CDK1 homologue, would be activated by Pfcyc-1, a protein that is most closely

related, at the primary structure level, to the cyclin H family and would therefore be expected to specifically activate a CDK7 homologue (for an extended discussion of this topic, see Ref. 5). Two of the cyclins described here clearly display primary structure relatedness to well defined classes of cyclins, as determined by BLAST analysis; the Pfcyc-2 sequence is more related to mitotic (A and B) cyclins (despite lacking a MRAIL motif; see above and Fig. 1A), and Pfcyc-4 appears to fall within the ania-6 family of cyclins (22). Members of the latter family appear to play a role in transcription control in some mammalian cell types and have been shown to associate with CDK-related kinases of the PITSLRE family (22). Several isoforms of PITSLRE kinases are found in mammalian cells and play a role in a variety of processes, including cell division (35) and transcriptional control (36). Pfcrk-1 is a putative malarial member of this kinase family (37), and it would be tempting to speculate that Pfcyc-4 may be an activating partner for this enzyme. However, in view of the unexpected observations (discussed above) concerning the cyclins capable of activating PfPK5 and considering the well known limitations of BLAST analysis-based protein classification (38), it would be dangerous to predict precise functions (and/or kinase subunit partners) for the cyclins described in this work on the sole basis of their sequence. Clearly, the functions of these elements must be explored experimentally. To this end, we envisage adopting the RNA interference approach, which has recently been applied successfully to essential plasmodial genes (39–41).

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**Identification and Initial Characterization of Three Novel Cyclin-related Proteins of the Human Malaria Parasite *Plasmodium falciparum***

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